

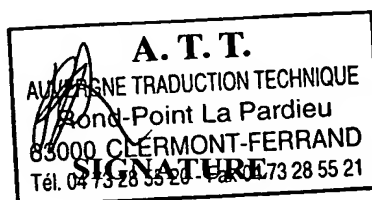
Auvergne Traduction Technique

**IN THE MATTER OF an Application  
for an Indian patent corresponding to  
PCT Application PCT/FR2004/02848.**

I, Richard Ryan, do solemnly  
and sincerely declare that I am conversant with the English and French  
languages and am a competent translator thereof, and that to the best of my  
knowledge and belief the following is a true and correct translation of the PCT  
Patent Application filed under No. PCT/FR2004/02848.

**For : Strains of microorganisms optimized for biosynthetic pathways  
consuming NADPH**

Date : 8-03-06



**Strains of microorganisms optimized for biosynthetic pathways consuming NADPH**

5 NADP (nicotinamide adenine dinucleotide phosphate), in its reduced form NADPH, takes part in intracellular redox reactions involving enzymes with dehydrogenase or reductase activities.

The present invention concerns strains of microorganisms optimized for the production, by biotransformation, of substances with NADPH-consuming biosynthesis routes. The strains according to the invention can be used in NADPH-consuming biotransformation processes. The strains defined according to the invention can be prokaryotic or eukaryotic. In a preferred embodiment, the prokaryotic strain is a strain of *Escherichia coli*. In a further embodiment the eukaryotic strain is a strain of *Saccharomyces*, in particular *S. cerevisiae*.

15 The present invention also concerns a process for the preparation of substances by biotransformation, through growth in an appropriate medium of a strain optimized according to the invention, which optimized strain also includes the genetic elements necessary for the preparation of such substances.

Biotransformation processes have been developed to allow the production of required substances in large quantities and at low cost, while at the same time making profitable use of various industrial or agricultural by-products.

20 There are two main approaches for producing substances of interest by *in vivo* biotransformation:

- 1) Fermentation, whereby a substance is produced by a microorganism from a simple carbon source (*e.g.* WO0102547, which describes the production of lysine by fermentation of *C. glutamicum* in the presence of glucose).
- 25 2) Bioconversion by a microorganism of a given co-substrate into a substance of interest (*e.g.* WO0012745, which describes the production of derivatives of R-piperidine, and WO0068397, which describes the production of tagatose). The co-substrate is not assimilated and is distinct from the carbon source, which is used solely to produce the biomass and the NADPH necessary for bioconversion.

The improvement of a biotransformation process can concern various factors such as temperature, oxygenation, medium composition, recovery process, etc. It can also be possible to modify a microorganism so as to increase the production and/or excretion of a substance of interest.

5           In a fermentation approach, for example, the biosynthesis route can be improved, for example, by modifying gene regulation or by modifying genes to change the characteristics of the enzymes involved, or by optimizing the regeneration of cofactors.

          In a bioconversion approach the emphasis will be placed on reducing the formation of by-products and optimizing the regeneration of the cofactors involved in the bioconversion  
10   step or steps.

          Among the cofactors involved in the biotransformations, NADPH is important in particular for the production of amino acids (*e.g.* arginine, proline, isoleucine, methionine, lysine), vitamins (*e.g.* pantothenate, phylloquinone, tocopherol), aromatics (*e.g.* WO9401564), polyols (*e.g.* xylitol), polyamines (*e.g.* spermidine), hydroxyesters (*e.g.* ethyl-4-chloro-3-  
15   hydroxybutyrate) and other high added-value substances.

          The present invention concerns a strain of microorganisms optimized for the production of substances with NADPH-consuming biosynthesis routes.

          Instead of seeking to optimize the NADPH/NADP<sup>+</sup> ratio in the microorganism for each biotransformation, the inventors chose to produce modified microorganisms in order to obtain  
20   different NADPH/NADP<sup>+</sup> ratios, which modified microorganisms were then used to carry out NADPH-consuming biotransformations.

          According to the invention a strain of microorganism is taken to mean a set of microorganisms of the same species comprising at least one microorganism of that species. Thus the characteristics described for the strain apply to each of the microorganisms of that  
25   strain. Similarly, the characteristics described for any one of the microorganisms of the strain apply to the entire set of the microorganisms of that strain.

          The microorganisms optimized according to the invention include bacteria, yeasts and filamentous moulds, and in particular bacteria and yeasts belonging to the following species: *Aspergillus sp.*, *Bacillus sp.*, *Brevibacterium sp.*, *Clostridium sp.*, *Corynebacterium sp.*,

*Escherichia sp.*, *Gluconobacter sp.*, *Penicillium sp.*, *Pichia sp.*, *Pseudomonas sp.*, *Rhodococcus sp.*, *Saccharomyces sp.*, *Streptomyces sp.*, *Xanthomonas sp.*, *Candida sp.*

The principle of optimization of the NADPH/NADP<sup>+</sup> ratio is described below for *E. coli* and *S. cerevisiae*. The same principle can be similarly applied to all microorganisms  
5 grown in aerobic conditions.

The principle of optimisation of the NADPH/NADP<sup>+</sup> ratio consists in limiting the enzyme activities involved in the oxidation of NADPH, and/or favouring the enzyme activities that allow the reduction of NADP<sup>+</sup>. The enzyme activities involved in the oxidation of NADPH are limited by reducing, and in particular by inactivating, those activities, especially  
10 activities such as quinone oxidoreductase and/or soluble transhydrogenase. The enzyme activities that favour the reduction of NADP<sup>+</sup> are enhanced by setting the carbon flux via the pentose phosphate cycle and/or by modifying the cofactor specificity of at least one enzyme so that it uses NADP in preference to NAD, its usual cofactor.

The strains optimized according to the invention are obtained by molecular biology  
15 methods. Those skilled in the art know the protocols used to modify the genetic character of microorganisms. These methods are documented and can be readily implemented by those skilled in the art (Sambrook *et al.*, 1989 Molecular cloning: a laboratory manual. 2<sup>nd</sup> Ed. Cold Spring Harbor Lab., Cold Spring Harbor, New York.).

The methods used to limit an enzyme activity consist in modifying the gene that  
20 expresses it by means of an appropriate method, for example by causing one or more mutations in the coding part of the gene concerned, or by modifying the promoter region, in particular by replacing it with a sequence that reduces the gene expression.

The methods used to inactivate an enzyme consist in inactivating the product of the expression of the gene concerned by means of an appropriate method, or in inhibiting the  
25 expression of the gene concerned, or in deleting at least a part of the gene concerned so that its expression is prevented (for example deleting part or all of the promoter region necessary for its expression), or so that the expression product loses its function (for example by deletion in the coding part of the gene concerned).

Preferentially, the deletion of a gene comprises the removal of that gene, and if required its replacement by a selection marker gene to facilitate the identification, isolation and purification of the strains optimized according to the invention.

The inactivation of a gene in *E. coli* is preferably carried out by homologous recombination (Datsenko K.A., Wanner B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* 97: 6640-6645). The principle of a protocol is briefly as follows: a linear fragment, obtained *in vitro*, comprising the two regions flanking the gene, and at least one selection gene located between these two regions (generally an antibiotic resistance gene), is introduced into the cell. This fragment thus contains an inactivated gene. Cells that have undergone a recombination event and integrated the introduced fragment are then selected by plating on a selective medium. Cells that have undergone a double recombination event, in which the wild gene has been replaced by the inactivated gene, are then selected. This protocol can be improved by using positive and negative selection systems, in order to speed up the detection of double recombination events.

The inactivation of a gene in *S. cerevisiae* is also performed preferentially by homologous recombination (Baudin *et al.*, *Nucl. Acids Res.* 21, 3329-3330, 1993; Wach *et al.*, *Yeast* 10, 1793-1808, 1994; Brachmann *et al.*, *Yeast.* 14:115-32, 1998).

The processes that favour an enzyme activity involve stabilizing the product of the expression of the gene concerned by appropriate means, for example by diminishing its sensitivity to allosteric effectors, or by enhancing the expression of the gene so as to increase the quantity of enzyme produced.

The overexpression of a gene can be achieved by replacing the promoter of the gene *in situ* by a strong or inducible promoter. Alternatively, a replicative plasmid (single or multiple copy) is introduced into the cell in which the gene to be overexpressed is under the control of the appropriate promoter. In the case of modification of *Escherichia coli*, it is possible, for example, to use the promoters *Plac-o*, *P<sub>trc</sub>-o*, and *ptac-o*, three strong bacterial promoters for which the lac operator (*lacO*) has been deleted to make them constitutive. In the case of modifications of *Saccharomyces cerevisiae*, it is possible, for example, to use the promoters *P<sub>pgk</sub>*, *P<sub>adh1</sub>*, *P<sub>gal1</sub>*, *P<sub>gal10</sub>*.

The processes that can be used to modify the cofactor specificity of an enzyme so that it uses NADP in preference to NAD involve modifying the sequence of the gene that allows the expression of that enzyme (Bocanegra, J.A. Scrutton, N.S.; Perham, R.N. (1993) Creation of an NADP-dependent pyruvate dehydrogenase multienzyme complex by protein engineering. *Biochemistry* **32**: 2737-2740).

The strains optimized according to the invention, *i.e.* with enhanced capacity for NADP<sup>+</sup> reduction, are characterized by the attenuation or inactivation of one or more NADPH-oxidizing enzyme activities, and in particular activities of the quinone oxidoreductase and/or soluble transhydrogenase type.

Below are listed some non-limiting examples of activities and genes of NADPH-oxidizing enzymes:

| Enzyme activity                                    | EC number  | <i>E. coli</i> gene | <i>S. cerevisiae</i> gene |
|--|------------|---------------------|---------------------------|
| Alcohol dehydrogenase                              | 1.1.1.2    | <i>yahK</i>         | ADH6                      |
| Aldose reductase                                   | 1.1.1.21   |                     | GRE3                      |
| Shikimate dehydrogenase                            | 1.1.1.25   | <i>aroE</i>         | ARO1                      |
| Methylglyoxal reductase                            | 1.1.1.78   |                     | GRE2p                     |
| Gamma-glutamyl phosphate reductase                 | 1.2.1.41   | <i>proA</i>         | PRO2                      |
| 2,4-dienoyl coenzyme A reductase                   | 1.3.1.34   | <i>fadH</i>         |                           |
| Glutamate dehydrogenase                            | 1.4.1.4    | <i>gdhA</i>         | GDH1, GDH2                |
| Glutamate synthase                                 | 1.4.1.13   | <i>gltB, gltD</i>   | GLT1                      |
| Methylenetetrahydrofolate dehydrogenase            | 1.5.1.5    | <i>folD</i>         | ADE3, MIS1                |
| Soluble transhydrogenase                           | 1.6.1.1    | <i>udhA</i>         |                           |
| Membrane-bound transhydrogenase                    | 1.6.1.2    | <i>pntA, pntB</i>   |                           |
| Quinone oxidoreductase                             | 1.6.5.5    | <i>qor</i>          | ZTA1                      |
| Nitrite reductase                                  | 1.7.1.4    | <i>nirB, nirD</i>   |                           |
| Sulphite reductase                                 | 1.8.1.2    | <i>cysI, cysJ</i>   |                           |
| Sterol demethylase                                 | 1.14.13.70 |                     | ERG11                     |
| 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase | 1.17.1.2   | <i>ispH</i>         |                           |
| Flavodoxin reductase                               | 1.18.1.2   | <i>fpr</i>          |                           |

The strains optimized according to the invention (*i.e.* with enhanced capacity for NADP<sup>+</sup> reduction) also include modifications that favour one or more NADP<sup>+</sup>-reducing enzyme activities, and in particular modifications that set the carbon flux via the pentose

phosphate pathway, and/or modifications concerning the cofactor specificity of at least one enzyme, so that it utilizes NADP in preference to NAD, its usual cofactor.

The activities that are susceptible to modification in the optimized strains according to the invention (*i.e.* with enhanced capacity for NADP<sup>+</sup> reduction) to favour one or more

5 NADP<sup>+</sup>-reducing enzyme activities are listed below:

| Enzyme activity                             | EC number | <i>E. coli</i> gene       | <i>S. cerevisiae</i> gene |
|---|-----------|---------------------------|---------------------------|
| Phosphoglucose isomerase                    | 5.3.1.9   | <i>pgi</i>                | PGI1                      |
| Phosphofructokinase                         | 2.7.1.11  | <i>pfkA</i> , <i>pfkB</i> | PFK1, PFK2                |
| Glucose 6-phosphate dehydrogenase           | 1.1.1.49  | <i>zwf</i>                | ZWF1                      |
| 6-Phosphogluconolactonase                   | 3.1.1.31  |                           | SOL1, SOL2, SOL3, SOL4    |
| 6-Phosphogluconate dehydrogenase            | 1.1.1.44  | <i>gnd</i>                | GND1, GND2                |
| 6-Phosphogluconate dehydratase              | 4.2.1.12  | <i>edd</i>                |                           |
| Malate synthase                             | 2.3.3.9   | <i>aceB</i>               | DAL7                      |
| Isocitrate lyase                            | 4.1.3.1   | <i>aceA</i>               | ICL1                      |
| Isocitrate dehydrogenase                    | 1.1.1.42  | <i>icd</i>                | IDP1, IDP2, IDP3          |
| Isocitrate dehydrogenase kinase/phosphatase | 2.7.1.116 | <i>aceK</i>               |                           |
| Dihydrolipoamide dehydrogenase              | 1.8.1.4   | <i>lpd</i>                | LPD1                      |
| Glyceraldehyde-3-phosphate dehydrogenase    | 1.2.1.12  | <i>gapA</i> , <i>gapC</i> | TDH1, TDH2, TDH3          |

The enzyme activities susceptible to modification in the optimized strains according to the invention are mainly defined using the denomination of the protein or gene in *E. coli* or *S. cerevisiae*. However, this usage has a more general meaning according to the invention, and covers the corresponding enzyme activities in other microorganisms. Using the sequences of proteins and genes in *E. coli* or *S. cerevisiae*, those skilled in the art can identify the equivalent genes in microorganisms other than *E. coli* or *S. cerevisiae*.

The means of identifying homologous sequences and their percentages of homology are well known to those skilled in the art, and include, in particular, the BLAST program, which can be used from the website <http://www.ncbi.nlm.nih.gov/BLAST/> with the default parameters indicated there. The sequences obtained can then be exploited (*e.g.* aligned) using, for example, the CLUSTALW program (<http://www.ebi.ac.uk/clustalw/>) or the MULTALIN program (<http://prodes.toulouse.inra.fr/multalin/cgi-bin/multalin.pl>), with the default parameters indicated on their websites.

Alternatively, the CD-Search program (<http://www.ncbi.nih.gov/Structure/cdd/wrpsb.cgi>) can be used to identify the conserved domains in the protein sequences of *E. coli* or *S. cerevisiae*, and to seek the sequences of other microorganisms presenting the same domain or domains. The conserved domains are recorded in the CDD database (Conserved domain database; Marchler-Bauer A, Anderson JB, DeWeese-Scott C, Fedorova ND, Geer LY, He S, Hurwitz DI, Jackson JD, Jacobs AR, Lanczycki CJ, Liebert CA, Liu C, Madej T, Marchler GH, Mazumder R, Nikolskaya AN, Panchenko AR, Rao BS, Shoemaker BA, Simonyan V, Song JS, Thiessen PA, Vasudevan S, Wang Y, Yamashita RA, Yin JJ, Bryant SH. CDD: a curated Entrez database of conserved domain alignments. Nucleic Acids Research 31:383-387 (2003)) which groups data of the PFAM or COG type.

The PFAMs (Protein FAMilies database of alignments and hidden Markov models; <http://www.sanger.ac.uk/Software/Pfam/>) are a large collection of alignments of protein sequences. Each PFAM makes it possible to visualize multiple alignments, see protein domains, evaluate distribution among organisms, access other databases, and visualize known protein structures.

The COGs (Clusters of Orthologous Groups of proteins; <http://www.ncbi.nlm.nih.gov/COG/>) are obtained by comparing protein sequences from 43 fully sequenced genomes representing 30 major phylogenetic lines. Each COG is defined from at least three lines, thus making it possible to identify ancient conserved domains.

From consensus sequences identified by these different methods, it is possible to design degenerate oligonucleotide probes to clone the corresponding gene in another microorganism. These routine molecular biology methods are well known to those skilled in the art and are described, for example, in Sambrook *et al.* (1989 Molecular cloning: a laboratory manual. 2<sup>nd</sup> Ed. Cold Spring Harbor Lab., Cold Spring Harbor, New York.).

Examples of genes coding for proteins analogous to the soluble transhydrogenase of *E. coli* coded for by the *udhA* gene:



| Gene        | Microorganism                        |
|-------------|--------------------------------------|
| <i>sth</i>  | <i>Azotobacter vinelandii</i>        |
| <i>udhA</i> | <i>Salmonella typhimurium</i> LT2    |
| <i>sthA</i> | <i>Pseudomonas aeruginosa</i> PA01   |
| <i>sth</i>  | <i>Pseudomonas fluorescens</i>       |
| <i>sthA</i> | <i>Pseudomonas putida</i> KT2440     |
| <i>udhA</i> | <i>Shigella flexneri</i> 2a str. 301 |
| <i>sthA</i> | <i>Vibrio cholera</i>                |
| <i>sthA</i> | <i>Yersinia pestis</i>               |

Examples of genes coding for proteins analogous to the quinone oxidoreductase of *E. coli* coded for by the *qor* gene:

| Gene        | Microorganism                            |
|-------------|--|
| <i>qor</i>  | <i>Bradyrhizobium japonicum</i> USDA 110 |
| <i>qor</i>  | <i>Brucella suis</i> 1330                |
| CC3759      | <i>Caulobacter crescentus</i>            |
| mll0505     | <i>Mesorhizobium loti</i>                |
| <i>qor</i>  | <i>Mycobacterium tuberculosis</i> H37RV  |
| <i>qor</i>  | <i>Pseudomonas aeruginosa</i>            |
| ZTA1        | <i>S. cerevisiae</i>                     |
| SPCC285.01c | <i>Schizosaccharomyces pombe</i>         |
| <i>drgA</i> | <i>Synechocystis</i> sp. PCC6803         |
| <i>qorA</i> | <i>Staphylococcus aureus</i>             |
| TTC0305     | <i>Thermus thermophilus</i> HB8          |
| <i>qor</i>  | <i>Yersinia pestis</i> CO92              |

5

The strains optimised according to the invention (*i.e.* with enhanced capacity for NADP<sup>+</sup> reduction) are characterized by the deletion of at least one gene coding for a NADPH-oxidizing activity, and in particular, the deletion of a gene coding for a quinone oxidoreductase (*e.g.* *qor*, ZTA1) and/or a gene coding for a soluble transhydrogenase activity  
10 (*e.g.* *udhA*).

In a preferred embodiment of the invention the *udhA* and *qor* genes are both deleted.

In a specific embodiment of the invention the strains optimized according to the invention are also characterized by the deletion of one or several genes coding for

phosphoglucose isomerase activity (*e.g. pgi*, PGI1) and/or phosphofructokinase activity (*e.g. pfkA*, PFK1).

In a further specific embodiment of the invention the strains optimized according to the invention are also characterized by the modification of one or several genes coding for  
5 dihydrolipoamide dehydrogenase (*e.g. lpd*, LPD1) and/or glyceraldehyde 3-phosphate dehydrogenase (*e.g. gapA*, TDH1) activities, which modification consists in causing the enzyme to prefer NADP over NAD, its usual cofactor.

The strains of the invention characterized by the deletion of genes coding for phosphoglucose isomerase and/or phosphofructokinase activities are particularly well suited to  
10 biotransformation processes.

To increase the quantity of NADPH available in the microorganisms optimized according to the invention, it can be advantageous to overexpress at least one gene coding for one of the following enzyme activities: glucose 6-phosphate dehydrogenase (*e.g. zwf*, ZWF1), 6-phosphogluconolactonase (*e.g. SOL1*), 6-phosphogluconate dehydrogenase (*e.g. gnd*,  
15 GND1), isocitrate dehydrogenase (*e.g. icd*, IDP1) and membrane-bound transhydrogenase (*e.g. pntA*), and/or to delete at least one gene coding for one of the following enzyme activities: phosphogluconate dehydratase (*e.g. edd*), malate synthase (*e.g. aceB*, DAL7), isocitrate lyase (*e.g. aceA*, ICL1) and isocitrate dehydrogenase kinase/phosphatase (*e.g. aceK*).

A further object of the present invention is a microorganism optimized for the  
20 production of NADPH as defined above and below, which microorganism also contains one or several genes coding for enzyme activities involved in the biotransformation of a substance of interest, and one or several selection marker genes.

These genes can be native to the strain optimized by the invention, or be introduced into the strain optimized by the invention by conversion using a suitable vector, either by  
25 integration in the genome of the microorganism or by a replicative vector, which suitable vector bears one or several genes coding for the relevant enzymes involved in the biotransformation of the relevant substance of interest and/or the relevant selection markers.

These genes include a nucleic acid sequence coding for an enzyme involved in the biotransformation of the substance of interest and/or for a selection marker, which coding  
30 sequence is merged with efficient promoter sequences in the prokaryote and/or eukaryote cell

selected for biotransformation. The vector (or plasmid) can be a shuttle vector between *E. coli* and another microorganism.

The choice of the strain optimized for the NADPH/NADP<sup>+</sup> ratio will be determined according to the type of biotransformation (fermentation or bioconversion), the total demand  
5 for NADPH in the bioconversion pathway considered, the nature of the carbon source or sources, the biomass flux demand, etc.

The deletion of genes coding for phosphoglucose isomerase and/or phosphofructokinase activities will be necessary if it is not possible to control the distribution of the carbon flux between glycolysis and the pentose phosphate pathway. The deletion of  
10 genes coding for phosphoglucose isomerase will be preferred for fermentations or when the demand for NADPH requires a minimum reduction flux of 2 moles of NADP<sup>+</sup> per mole of imported glucose. The deletion of genes coding for phosphofructokinase will be chosen preferentially for bioconversions or when the demand for NADPH requires a minimum  
15 reduction flux of 3-4 moles of NADP<sup>+</sup> per mole of imported glucose. The modification, as described above and below, of genes coding for dihydrolipoamide dehydrogenase and/or glyceraldehyde 3-phosphate dehydrogenase will be carried out when biotransformations require a minimum reduction flow greater than 3 moles of NADP<sup>+</sup> per mole of imported  
20 glucose and in particular to optimize the strains *E. coli*  $\Delta(udhA, qor)$  or *E. coli*  $\Delta(udhA, qor, pgi)$  or *E. coli*  $\Delta(udhA, qor, pfkA, pfkB)$ . The other stated modifications, namely the overexpression of at least one gene coding for one of the following enzyme activities: glucose  
25 6-phosphate dehydrogenase, 6-phosphogluconolactonase, 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase and membrane transhydrogenase, and/or deletion of at least one gene coding for one of the following enzyme activities: 6-phosphogluconate dehydratase, malate synthase, isocitrate lyase or isocitrate dehydrogenase kinase/phosphatase, can be carried out to  
fine-tune the optimization of the NADPH/NADP<sup>+</sup> ratio to the needs of the cell and of the biotransformation process being considered.

The present invention also concerns a procedure for preparing strains optimized according to the invention as defined above and below, characterized by the deletion of a gene coding for quinone oxidoreductase or soluble transhydrogenase activities, and possibly by the

deletion of a gene coding for glucose 6-phosphate dehydrogenase or 6-phosphogluconolactonase activities, and/or by the modification of at least one gene coding for NAD enzymes, in particular for dihydrolipoamide dehydrogenase or glyceraldehyde 3-phosphate dehydrogenase, so that they preferentially use NADP, and if required, by the deletion of at least one gene coding for 6-phosphogluconate dehydratase, malate synthase, isocitrate lyase or isocitrate dehydrogenase kinase/phosphatase, which deletions and modifications are carried out by suitable means, and/or characterized by the overexpression of at least one gene coding for the following activities: glucose 6-phosphate dehydrogenase, 6-phosphogluconolactonase, 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase or membrane transhydrogenase, either by modifying the strain using a suitable vector that allows the overexpression, or by modifying the strength of the endogenous promoter controlling the gene that is to be overexpressed.

In a specific embodiment of the invention, the process for preparing strains according to the invention also includes the conversion of the optimized strains with at least one suitable vector that includes one or more genes coding for one or more enzymes involved in the biotransformation of a substance of interest, and one or more selection marker genes.

A further object of the invention concerns the use of these strains optimized according to the invention for NADPH-dependent biotransformations, thereby obtaining an improved biotransformation yield compared with a strain not optimized for NADPH.

The biotransformations will be carried out using strains defined according to the invention in which genes will be expressed that code for enzyme activities catalyzing NADPH-dependent reactions. Those skilled in the art can easily identify such enzymes. They include the following among others: EC 1.1.1.10 L-xylulose reductase, EC 1.1.1.21 methylglyoxal reductase, EC 1.1.1.51 3(or 17) $\beta$ -hydroxysteroid dehydrogenase, EC 1.1.1.54 allyl-alcohol dehydrogenase, EC 1.1.1.80 isopropanol dehydrogenase, EC 1.1.1.134 dTDP-6-deoxy-L-talose 4-dehydrogenase, EC 1.1.1.149 20 $\alpha$ -hydroxysteroid dehydrogenase, EC 1.1.1.151 21-hydroxysteroid dehydrogenase, EC 1.1.1.189 prostaglandin-E<sub>2</sub> 9-reductase, EC 1.1.1.191 indole-3-acetaldehyde reductase EC 1.1.1.207 (-)-menthol dehydrogenase, EC 1.1.1.234 flavanone 4-reductase, EC 1.2.1.50 long-chain-fatty-acyl-CoA reductase, EC 1.3.1.4 cortisone  $\alpha$ -reductase, EC 1.3.1.23 cholestenone 5 $\beta$ -reductase, EC 1.3.1.70  $\Delta^{14}$ -sterol

reductase, EC 1.4.1.12 2,4-diaminopentanoate dehydrogenase, EC 1.5.1.10 saccharopine dehydrogenase, L-glutamate-forming, EC 1.7.1.6 azobenzene reductase, EC 1.8.1.5 2-oxopropyl-CoM reductase (carboxylating), EC 1.10.1.1 *trans*-acenaphthene-1,2-diol dehydrogenase, EC 1.14.13.7 phenol 2-monooxygenase, EC 1.14.13.12 benzoate 4-mono-  
 5 oxygenase, EC 1.14.13.26 phosphatidylcholine 12-mono-oxygenase, EC 1.14.13.64 4-hydroxybenzoate 1-hydroxylase, EC 1.14.13.70 sterol 14-demethylase, EC 1.16.1.5 aquacobalamine reductase, EC 1.17.1.1 CDP-4-dehydro-6-deoxyglucose reductase, EC 1.18.1.2 ferredoxin-NADP reductase.

The invention also concerns a process for producing a substance of interest formed by  
 10 a biosynthesis route that includes at least one NADPH-dependent reaction, characterized in that it comprises the following steps:

- a) Growth in culture of microorganisms optimized according to the invention in an appropriate culture medium that favours their growth and that contains the substances necessary to carry out the biotransformation by fermentation or bioconversion, except  
 15 NADPH.
- b) Extraction of the substance of interest from the medium and its purification if necessary.

Preferably, the substance of interest can be an amino acid, a vitamin, a sterol, a flavonoid, a fatty acid, an organic acid, a polyol, or a hydroxyester. Amino acids and their  
 20 precursors include in particular lysine, methionine, threonine, proline, glutamic acid, homoserine, isoleucine, and valine. Vitamins and their precursors include in particular pantoate, trans-neurosporene, phylloquinone and tocopherols. Sterols include in particular squalene, cholesterol, testosterone, progesterone and cortisone. Flavonoids include in particular frambinone and vestitone. Organic acids include coumaric acid and 3-  
 25 hydroxypropionic acid. Polyols include sorbitol, xylitol and glycerol. Hydroxyesters include ethyl-3-hydroxybutyrate and ethyl-4-chloro-3-hydroxybutyrate.

In the case of a bioconversion, the process also includes the addition to the appropriate culture medium of the substrate that is to be converted.

The culture medium mentioned in step b) of the process according to the invention  
 30 described above contains at least one assimilable carbohydrate that can be any of various

assimilable sugars, such as glucose, galactose, sucrose, lactose, molasses, or by-products of these sugars. A simple source of carbon that is especially preferred is glucose. Another preferred simple carbon source is sucrose. The culture medium can also contain one or more substances (*e.g.* amino acids, vitamins or mineral salts) that favour the growth of the microorganism and/or the production of the substance of interest. In particular, the mineral culture medium for *E. coli* can thus have a composition identical or similar to an M9 medium (Anderson, 1946, *Proc. Natl. Acad. Sci. USA* **32**:120-128), an M63 medium (Miller, 1992; A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) or a medium such as that described by Schaefer *et al.* (1999, *Anal. Biochem.* **270**: 88-96).

The biotransformation conditions are set by those skilled in the art. In particular, the microorganisms are fermented at a temperature between 20°C and 55°C, preferably between 25°C and 40°C, and preferably at about 30°C for *S. cerevisiae* and at about 37°C for *E. coli*.

The following examples are given for illustration only and in no way restrict the embodiments or the scope of the invention.

### **Example 1: Calculation of theoretical optimal yields for the bioconversion of ethylacetoacetate into ethyl-3-hydroxybutyrate**

#### **a) Bioconversion with *E. coli***

Predictive modelling was carried out using the algorithm MetOpt®-Coli, a stoichiometric model developed by the company METabolic EXplorer, with which it was possible to determine 1) the maximum production yield of ethyl-3-hydroxybutyrate from ethylacetoacetate, and 2) the best flux distribution from glucose to meet the needs for growth and redox equilibria that are necessary for the cell to grow and reach the maximum bioconversion yield.

Specific settings of model variables were 1) a glucose import flux of 3 mmol.g<sup>-1</sup>.h<sup>-1</sup>, 2) a variable growth rate of 0, 0.15 and 0.25 h<sup>-1</sup>, 3) a variable membrane-bound transhydrogenase flux (*pntAB*) less than or equal to 1 mmol.g<sup>-1</sup>.h<sup>-1</sup>; the limiting value of the membrane-bound transhydrogenase flux was determined from the literature (Hanson, 1979; Anderlund *et al.*,

1999; Emmerling *et al.*, 2002), and 4) a maintenance flux limited at between 5 and 22 mmol.g<sup>-1</sup>.h<sup>-1</sup>.

In all cases the model suggested the deletion of the *udhA* and *qor* genes. In practice, however, the strain *E. coli* [ $\Delta(\textit{udhA}, \textit{qor})$ ] does not afford a yield equivalent to the theoretical optimal yield, as it is difficult to maintain a suitable distribution of carbon flux between the pentose phosphate pathway and glycolysis, this distribution being variable according to the growth rate. In practice therefore, the strains *E. coli* [ $\Delta(\textit{udhA}, \textit{qor}, \textit{pfkA}, \textit{pfkB})$ ] or [ $\Delta(\textit{udhA}, \textit{qor}, \textit{pgi})$ ] are preferred, the choice between them depending on the growth rate of the strain during the bioconversion process.

The theoretical optimal yields for the bioconversion of ethylacetoacetate into ethyl-3-hydroxybutyrate were calculated for different strains of *E. coli* optimized according to the invention.

|  | $\mu = 0$ | $\mu = 0.15 \text{ h}^{-1}$ | $\mu = 0.25 \text{ h}^{-1}$ |
|--|-----------|-----------------------------|-----------------------------|
| $\Delta(\textit{udhA}, \textit{qor}, \textit{pgi})$  | 1.82      | 1.74                        | 1.22                        |
| $\Delta(\textit{udhA}, \textit{qor}, \textit{pgi})$<br><i>gapA</i> -NADP-dependent   | 4.29      | 3.64                        | 2.43                        |
| $\Delta(\textit{udhA}, \textit{qor}, \textit{pgi})$<br><i>lpd</i> -NADP-dependent  | 5.67      | 3.46                        | 1.99                        |
| $\Delta(\textit{udhA}, \textit{qor}, \textit{pgi})$<br><i>gapA</i> -NADP-dependent<br><i>lpd</i> -NADP-dependent                 | 6.86      | 4.96                        | 3.33                        |
| $\Delta(\textit{udhA}, \textit{qor}, \textit{pfkA}, \textit{pfkB})$  | 6.76      | 4.65                        | 0.19                        |
| $\Delta(\textit{udhA}, \textit{qor}, \textit{pfkA}, \textit{pfkB})$<br><i>gapA</i> -NADP-dependent                               | 8.16      | 5.54                        | 1.02                        |
| $\Delta(\textit{udhA}, \textit{qor}, \textit{pfkA}, \textit{pfkB})$<br><i>lpd</i> -NADP-dependent                                | 8.33      | 5.60                        | 1.77                        |
| $\Delta(\textit{udhA}, \textit{qor}, \textit{pfkA}, \textit{pfkB})$<br><i>gapA</i> -NADP-dependent<br><i>lpd</i> -NADP-dependent | 9.33      | 6.38                        | 2.60                        |

**Theoretical optimal yields for the bioconversion of ethylacetoacetate into ethyl-3-hydroxybutyrate (mol per mol of glucose) by strains of *E. coli* optimized for NADP<sup>+</sup> reduction capacity**

To further improve the theoretical optimal yield of the strains optimized according to the invention, additional modifications can be made, such as the overexpression of at least one gene that can be *zwf*, *gnd*, *pntA*, *pntB* or *icd* and/or the deletion of at least one gene that can be *edd*, *aceA*, *aceB* or *aceK*.

**b) Bioconversion with *S. cerevisiae***

Predictive modelling was carried out using the algorithm MetOpt®-Scere, a stoichiometric model developed by the Company, with which it was possible to determine 1) the maximum production yield of ethyl-3-hydroxybutyrate from ethylacetoacetate, 2) the best flux distribution from glucose to meet the needs of the growth and redox equilibria necessary for the cell to grow and reach the maximum bioconversion yield.

Specific settings of model variables were 1) a glucose import flux of 3 mmol.g<sup>-1</sup>.h<sup>-1</sup>, 2) a variable growth rate of 0, 0.15 and 0.25 h<sup>-1</sup>, 3) a maintenance flux de maintenance less than or equal to 22 mmol.g<sup>-1</sup>.h<sup>-1</sup>, 4) aldehyde dehydrogenase reactions (ALD2, ALD3, ALD6) irreversible and set in the direction acetate + NAD(P)H → acetaldehyde + NAD(P), and 5) no activities equivalent to *udhA* or *pntA,B*.

The model allowed for mitochondrial and peroxisomal compartmentalization.

In all cases, the model suggested the deletion of a gene coding for an enzyme oxidizing NADPH, and in particular gene ZTA1. However, in practice, the strain *S. cerevisiae* [ $\Delta$ ZTA1] does not provide a yield equivalent to the theoretical optimum yield, as it is difficult to maintain a suitable distribution of carbon flux between the pentose phosphate and glycolysis pathways, because this distribution varies with the growth rate. In practice it is preferable to use the strains *S. cerevisiae* [ $\Delta$ (ZTA1, PFK1, PFK2)] or [ $\Delta$ (ZTA1, PGI1)], the choice between them depending on rate of growth of the strain during the bioconversion process.

The theoretical optimal yields for the bioconversion of ethylacetoacetate into ethyl-3-hydroxybutyrate were calculated for different strains of *S. cerevisiae* optimized according to the invention.



|   | $\mu = 0$ | $\mu = 0.15 \text{ h}^{-1}$ | $\mu = 0.25 \text{ h}^{-1}$ |
|---|-----------|-----------------------------|-----------------------------|
| $\Delta(\text{ZTA1,PGI1})$  | 2.42      | 2.00                        | 1.73                        |
| $\Delta(\text{ZTA1,PGI1})$<br>TDH1,2,3-NADP-dependent                               | 4.22      | 3.50                        | 3.03                        |
| $\Delta(\text{ZTA1,PGI1})$<br>LPD1-NADP-dependent                                   | 4.08      | 3.29                        | 2.77                        |
| $\Delta(\text{ZTA1,PGI1})$<br>TDH1,2,3-NADP-dependent<br>LPD1-NADP-dependent        | 6.17      | 5.01                        | 4.23                        |
| $\Delta(\text{ZTA1, PFK1, PFK2})$   | 12.00     | 8.18                        | 5.64                        |
| $\Delta(\text{ZTA1, PFK1, PFK2})$<br>TDH1,2,3-NADP-dependent                        | 12.00     | 9.11                        | 7.19                        |
| $\Delta(\text{ZTA1, PFK1, PFK2})$<br>LPD1-NADP-dependent                            | 12.00     | 8.44                        | 6.06                        |
| $\Delta(\text{ZTA1, PFK1, PFK2})$<br>TDH1,2,3-NADP-dependent<br>LPD1-NADP-dependent | 12.00     | 9.28                        | 7.46                        |

**Theoretical optimal yields for the bioconversion of ethylacetoacetate into ethyl-3-hydroxybutyrate (mol per mol of glucose) by strains of *S. cerevisiae* optimized for NADP<sup>+</sup> reduction capacity**

To further improve the theoretical optimum yield of the strains optimized according to the invention, additional modifications can be made, such as the overexpression of at least one gene that can be ZWF, SOL1, SOL2, SOL3, SOL4, GND1, GND2, IDP1, IDP2 or IDP3 and/or the deletion of at least one gene that can be either ICL1 or DAL7.

**Example 2: Construction of the strain *E. coli* [ $\Delta(\text{udhA, qor})$ ]**

The inactivation of the *udhA* gene was achieved by homologous recombination using the method described by Datsenko and Wanner (One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products, *Proc. Natl. Acad. Sci. USA*, 2000, 97: 6640-6645).

This method consists in inserting an antibiotic (chloramphenicol) resistance cassette while at the same time deleting most of the gene concerned. For this purpose a pair of oligonucleotides was synthesized, each consisting of 100pb of which 80pb (lower case) were

homologous with the gene to be deleted (*e.g. udhA*) and 20pb (upper case) were homologous with the chloramphenicol resistance cassette.

DudhAF

ggtgcgcgcgtcgagttatcgagcgttatcaaaatgttggcggcggtgcacccactggggcaccatcccgtcgaaagcCATAT

5 GAATATCCTCCTTAG

DudhAR

CccagaatctctttgtttcccgatggaacaaaatttcagcgtgccacgttcacgacgattgtgcgcgtgccagTGTAGGC  
TGGAGCTGCTTCG

The antibiotic cassette carried by the plasmid pKD3 was amplified by PCR using the  
10 oligonucleotides DudhAF and DudhAR. The PCR product obtained was then introduced by electroporation into the strain *E. coli* [pKD46], which carries the gene coding for Red recombinase, an enzyme that catalyzes homologous recombination. The chloramphenicol-resistant transformants were then selected and the insertion of the resistance cassette was checked by PCR analysis using the oligonucleotides UdhAF and UdhAR:

15 UdhAF

Ggccgctcaggatatagccagataaatgac

UdhAR

Gcgggatcactttactgccagcgctggctg

The chloramphenicol resistance cassette was then removed. To do this, the plasmid  
20 pCP20 carrying the FLP recombinase acting on the FRT sites of the chloramphenicol resistance cassette was introduced into the recombinant strains by electroporation. After a series of cultures at 42°C, the loss of the antibiotic resistance cassette was checked by PCR analysis with the oligonucleotides UdhAF and UdhAR.

The inactivation of the gene *qor* was carried out by the same method using the  
25 following oligonucleotides:

DqorF

ggtggcccggaagtacttcaagccgtagagttcactcctgccgatccggcggaatgaaatccaggtcgaaaataaagcCATAT  
GAATATCCTCCTTAG

DqorR

cgcccggtttccagaatctcatgcgacgctgcgcacgttcagcggatatttctgctgctggcgacatcgaccttaaTG TAGGC  
TGGAGCTGCTTCG

QorF

Cgccaacaccgactgctccgcttcgatcg

5 QorR

cagcggtatgaccgctggcggttactaaggg

For practical reasons it can be useful to delete the two genes simultaneously. To do this each gene was replaced by a different antibiotic resistance cassette (e.g. chloramphenicol for *udhA* and kanamycin for *qor*).

10 The strain obtained was thus *E. coli* [ $\Delta$ (*udhA*, *qor*)].

**Example 3: Construction of the plasmid pSK-PgapA-GRE2p,  
introduction into the strain *E. coli* [ $\Delta$ (*udhA*, *qor*)] and bioconversion of ethylacetoacetate  
into ethyl-3-hydroxybutyrate**

15 The plasmid pSK-PgapA was constructed by insertion of the promoter *gapA* in the vector pBluescript-SK (pSK). To do this, the promoter *gapA* of *E. coli* was amplified with polymerase *Pwo* from chromosomal DNA.

The PCR product obtained was then digested by the restriction enzyme *Hind*III and ligated to the vector pSK digested by the restriction enzyme *Hind*III and dephosphorylated to  
20 give the plasmid pSK-PgapA. The vector pSK carried a replication origin for *E. coli* and an ampicillin resistance gene.

The plasmid pSK-PgapA was then introduced into the strain *E. coli* DH5 $\alpha$  for verification of the construction. The sequencing of the promoter *gapA* of the plasmid pSK-PgapA with the oligonucleotide universal M13 forward was then carried out to confirm the  
25 construction.

The plasmid pSK-PgapA-GRE2p was constructed by insertion of the gene GRE2p in the plasmid pSK-PgapA. To do this, the gene GRE2p of *Saccharomyces cerevisiae* was amplified with polymerase *Pwo* from chromosomal DNA using the following oligonucleotides:

30

Ome119\_GRE2F (NdeI)

Acgtacgtggcatatgtcagttttcgttcaggtgctaacggg

Ome120\_GRE2R (PstI)

Acgtacctgcagttatattctgccctcaaattttaaaatttggg

5           The PCR product obtained was then digested by restriction enzymes *NdeI* - *PstI* and ligated to the vector pSK-PgapA digested by the restriction enzymes *NdeI* - *PstI* and dephosphorylated to give the plasmid pSK-PgapA-GRE2p. The plasmid pSK-PgapA carried a replication origin for *E. coli* and an ampicillin resistance gene.

10           The plasmid pSK-PgapA-GRE2p was then introduced into the strain *E. coli* DH5α to check the construction. The sequencing of the gene GRE2p of the plasmid pSK-PgapA-GRE2p with the oligonucleotide universal M13 reverse and universal M13 forward was then carried out to confirm the construction.

          The validated plasmid was introduced into the strain *E. coli* [ $\Delta(udhA, qor)$ ] (Example 2) by electroporation.

15           The strain obtained, *E. coli* [ $\Delta(udhA, qor)$  pSK-PgapA-GRE2p], was then grown in minimum medium containing glucose and ethylacetoacetate. A strain of *E. coli* [pSK-PgapA-GRE2p] was grown in the same conditions.

          When growth was completed the following variables were compared:

- The time course of the biomass of each strain during the bioconversion phase.
- 20    - The quantity of ethyl-3-hydroxybutyrate produced in the extracellular medium.
- The quantity of ethyl-3-hydroxybutyrate accumulated in the cells.
- The productivity in terms of ethyl-3-hydroxybutyrate.
- The yield glucose/ethyl-3-hydroxybutyrate.

25           It was found that the strain *E. coli* [ $\Delta(udhA, qor)$  pSK-PgapA-GRE2p] gave a greater production yield of ethyl-3-hydroxybutyrate than the non-optimized strain.

**Example 4: Construction of the strain *E. coli* [ $\Delta(udhA, qor, pgi)$  pSK-PgapA-GRE2p] and bioconversion of ethylacetoacetate into ethyl-3-hydroxybutyrate**

The inactivation of the gene *pgi* was carried out in the strain *E. coli* [ $\Delta(udhA, qor)$ ] (Example 2) using the method described in Example 2, and the following oligonucleotides:

DpgiF

ccaacgcagaccgctgcctggcaggcactacagaaacacttcgatgaaatgaaagacgttacgatcgccgatcttttgcTGTAGG

5 CTGGAGCTGCTTCG

DpgiR

gcgccacgctttatagcggtaatacagaccattggctgagctatcgaggctgctgattctttatcatctttcagctctgCATATGAAT

ATCCTCCTTAG

pgiF

10 gcggggcggttgtaacgatggggtcatgc

pgiR

cggtatgattccgttaaattacagacaag

The construction was carried out in rich medium (*e.g.* LB). The plasmid pSK-PgapA-GRE2p was then introduced into the strain obtained (Example 3) by electroporation, and the resulting strain *E. coli* [ $\Delta(udhA, qor, pgi)$  pSK-PgapA-GRE2p] was selected on rich medium.

The strain obtained was then grown on minimum medium containing glucose and ethylacetoacetate. A strain of *E. coli* [pSK-PgapA-GRE2p] was grown under the same conditions.

When growth was completed the following variables were compared:

- 20 - The time course of the biomass of each strain during the bioconversion phase.
- The quantity of ethyl-3-hydroxybutyrate produced in the extracellular medium.
- The quantity of ethyl-3-hydroxybutyrate accumulated in the cells.
- The productivity in terms of ethyl-3-hydroxybutyrate.
- The yield glucose/ethyl-3-hydroxybutyrate.

25 We observed that the strain *E. coli* [ $\Delta(udhA, qor, pgi)$  pSK-PgapA-GRE2p] gave a greater production yield of ethyl-3-hydroxybutyrate than the non-optimized strain.

|   | mol <sub>EHB</sub> /mol <sub>Glucose</sub> |
|---|--|
| MG1655 pSK-PgapA-GRE2p                          | 0.75                                       |
| MG1655 $\Delta(udhA, qor, pgi)$ pSK-PgapA-GRE2p | 2.12                                       |

**Example 5: Construction of the strain *E. coli* [ $\Delta$ (*udhA*, *qor*, *pgi*, *edd*) pSK-PgapA-GRE2p] and bioconversion of ethylacetoacetate into ethyl-3-hydroxybutyrate**

The inactivation of the gene *edd* was carried out in the strain *E. coli* [ $\Delta$ (*udhA*, *qor*, *pgi*)] (Example 4) using the method described in Example 2, and the following

5 oligonucleotides:

DeddF (1932582-1932499)

CgcgcgagactcgctctgcttatctcgcccggatagaacaagcgaaaacttcgaccgttcacgttcgcagttggcatgcggTGTA  
GGCTGGAGCTGCTTCG

DeddR (1930866-1930943)

10 cgcaaggcgtgaataattcacgtcctgttcccacgcgtgacgcgctcaggtcaggaatgtcgggttcgcgagcagccCATATG  
AATATCCTCCTTAG

EddF (1932996-1932968)

Gggtagactccattactgaggcgtgggcg

EddR (1930439-1930462)

15 Ccccggaatcagaggaatagtc

The construction was carried out in rich medium (*e.g.* LB). The plasmid pSK-PgapA-GRE2p was then introduced into the strain obtained *E. coli* [ $\Delta$ (*udhA*, *qor*, *pgi*, *edd*)] (Example 3) by electroporation, and the resulting strain *E. coli* [ $\Delta$ (*udhA*, *qor*, *pgi*, *edd*) pSK-PgapA-GRE2p] was selected on rich medium.

20 The strain obtained *E. coli* [ $\Delta$ (*udhA*, *qor*, *pgi*, *edd*) pSK-PgapA-GRE2p] was then grown in a minimum medium containing glucose and ethylacetoacetate. A strain *E. coli* [pSK-PgapA-GRE2p] was grown under the same conditions.

When growth was completed the following variables were compared:

- The time course of the biomass of each strain during the bioconversion phase.
- 25 - The quantity of ethyl-3-hydroxybutyrate produced in the extracellular medium.
- The quantity of ethyl-3-hydroxybutyrate accumulated in the cells.
- The productivity in terms of ethyl-3-hydroxybutyrate.
- The yield glucose/ethyl-3-hydroxybutyrate.

We observed that the strain *E. coli* [ $\Delta(udhA, qor, pgi, edd)$  pSK-PgapA-GRE2p] gave a greater production yield of ethyl-3-hydroxybutyrate than the non-optimized strain.

**Example 6: Construction of the strain *E. coli* [ $\Delta(udhA, qor, pfkA, pfkB)$  pSK-PgapA-GRE2p] and bioconversion of ethylacetoacetate into ethyl-3-hydroxybutyrate**

The inactivation of the genes *pfkA* and *pfkB* was carried out in the strain *E. coli* [ $\Delta(udhA, qor)$ ] (Example 2) using the method described in Example 2 and the following oligonucleotides:

DpfkAF

10 ggt gtg ttg aca agc ggc ggt gat gcg cca ggc atg aac gcc gca att cgc ggg gtt gtt cgt tct gcg ctg  
aca gaa ggTGTAGGCTGGAGCTGCTTCG

DpfkAR

TtcgcgcagtcacagccagtcacctttgaacggacgttcattgttttcgatagcgtcgatgatgtcgtggtgaaccagctgCATATG  
AATATCCTCCTTAG

15 PfkAF

Cgcacgcggcagtcagggccgacccgc

PfkAR

ccctacgccccacttgttcacgccccg

DpfkBF (1804421-1804499)

20 gcgcctctctcgatagcgcaacaattaccccgcaatttatcccgaaggaaaactgcgctgtaccgcaccgggtgttcgTGTAGG  
CTGGAGCTGCTTCG

DpfkBR (1805320-1805241)

gcgggaaaggtaagcgtaaattttgcgtatcgatgggagcacagacgtgttcctgattgagtggtgcactccCATATG  
AATATCCTCCTTAG

25 PfkBF (1803996-1804025)

tggcaggatcatccatgacagtaaaaacgg

PfkBR (1805657-1805632)

gccgggtgcactttgggtaagccccg

The construction was carried out in rich medium (e.g. LB). The plasmid pSK-PgapA-  
30 GRE2p was then introduced into the strain obtained *E. coli* [ $\Delta(udhA, qor, pfkA, pfkB)$ ]

(Example 3) by electroporation, and the resulting strain *E. coli* [ $\Delta(udhA, qor, pfkA, pfkB)$  pSK-PgapA-GRE2p] was selected on rich medium.

The strain obtained *E. coli* [ $\Delta(udhA, qor, pfkA, pfkB)$  pSK-PgapA-GRE2p] was then grown in minimum medium containing glucose and ethylacetoacetate. A strain *E. coli* [pSK-PgapA-GRE2p] was grown under the same conditions.

When growth was completed the following variables were compared:

- The time course of the biomass of each strain during the bioconversion phase.
- The quantity of ethyl-3-hydroxybutyrate produced in the extracellular medium.
- The quantity of ethyl-3-hydroxybutyrate accumulated in the cells.
- The productivity in terms of ethyl-3-hydroxybutyrate.
- The yield glucose/ethyl-3-hydroxybutyrate.

We observed that the strain *E. coli* [ $\Delta(udhA, qor, pfkA, pfkB)$  pSK-PgapA-GRE2p] gave a greater production yield of ethyl-3-hydroxybutyrate than the non-optimized strain.

|  | mol <sub>EHB</sub> /mol <sub>Glucose</sub> |
|--|--|
| MG1655 pSK-PgapA-GRE2p                                 | 0.75                                       |
| MG1655 $\Delta(udhA, qor, pfkA, pfkB)$ pSK-PgapA-GRE2p | 3.46                                       |

**Example 7: Construction of the strain *E. coli* [ $\Delta(udhA, qor, pgi, lpd)$  *plpd\**, pSK-PgapA-GRE2p] and bioconversion of ethylacetoacetate into ethyl-3-hydroxybutyrate**

The gene *lpd* coding for NAD-dependent dihydrolipoamide dehydrogenase involved in the multienzyme complex pyruvate dehydrogenase was deleted using the method described in Example 2, except that the initial strain was the strain *E. coli* [ $\Delta(udhA, qor, pgi)$ ] described in Example 4 instead of being a wild strain. The construction and the selection of the modified strain were carried out in rich medium (*e.g.* LB). The strain obtained was *E. coli* [ $\Delta(udhA, qor, pgi, lpd)$ ].

In addition, the plasmid *p-lpd\** was constructed, which allows the overexpression of a NADP-dependent dihydrolipoamide dehydrogenase. There are various possible ways to modify the cofactor specificity of an enzyme. For example, Bocanegra *et al.* (1993) report a method to create a NADP-dependent dihydrolipoamide dehydrogenase.



The plasmids p-*lpd*\* and pSK-PgapA-GRE2p were then introduced by electroporation into the strain *E. coli* [ $\Delta(udhA, qor, pgi, lpd)$ ]. Alternatively, *lpd*\* could be cloned on pSK-PgapA-GRE2p, giving the plasmid pSK-PgapA-GRE2p-*lpd*\* then introduced by electroporation into the strain *E. coli* [ $\Delta(udhA, qor, pgi, lpd)$ ]. The construction and the  
 5 selection of the modified strain were carried out in rich medium (e.g. LB).

The strain *E. coli* [ $\Delta(udhA, qor, pgi, lpd)$  pSK-PgapA-GRE2p, p-*lpd*\*] obtained was then grown in minimum medium containing glucose and ethylacetoacetate. A strain *E. coli* [pSK-PgapA-GRE2p] was grown under the same conditions.

When growth was completed the following variables were compared:

- 10 - The time course of the biomass of each strain during the bioconversion phase.
- The quantity of ethyl-3-hydroxybutyrate produced in the extracellular medium.
- The quantity of ethyl-3-hydroxybutyrate accumulated in the cells.
- The productivity in terms of ethyl-3-hydroxybutyrate.
- The yield glucose/ethyl-3-hydroxybutyrate.

15 We observed that the strain *E. coli* [ $\Delta(udhA, qor, pgi, lpd)$  pSK-PgapA-GRE2p, p-*lpd*\*] gave a greater production yield of ethyl-3-hydroxybutyrate than the non-optimized strain.

#### **Example 8: Construction of the plasmid pRSGK-GRE2p**

20 The plasmid pYGK was constructed by insertion of the promoter *Ppgk*, the multicloning site and the terminator *cycl* of the vector pYPG2 in the vector pBluescript-SK (pSK). To do this, the promoter *Ppgk*, the multicloning site and the terminator *cycl* were amplified with polymerase *Pfu Turbo* from the vector pYPG2. The PCR product obtained was then digested by restriction enzymes *SacII* - *NotI* and ligated to the vector pSK digested by the  
 25 restriction enzymes *ApaI* – *SmaI*, ligated and digested by the restriction enzymes *NotI* – *SacII*, and dephosphorylated to give the plasmid pYGK.

The plasmid pYGK was then introduced into the strain *E. coli* DH5 $\alpha$  to verify the construction. The sequencing of the promoter *Ppgk*, the multicloning site and the terminator *cycl* of the plasmid pYGK with the oligonucleotides universal M13 reverse and universal  
 30 M13 forward was then carried out to confirm the construction.

The plasmid pYGK-GRE2p was then constructed by insertion of the gene GRE2p in the plasmid pYGK. To do this, the gene GRE2p of *Saccharomyces cerevisiae* was amplified with the polymerase *Pwo* from chromosomal DNA, using the following oligonucleotides:

Ome376\_Gre2 pYGK F (SmaI)

5 Acgtacgtcccccgggaaaaatgtcagttttcgtttcaggtgc

Ome377\_Gre2 pYGK R (ApaI)

ACGTACGGGCCCTTATATTCTGCCCTCAAATTTTAAAATTTGGG

The PCR product obtained was then digested by the restriction enzymes *ApaI* - *SmaI* and ligated to the vector pYGK digested by the restriction enzymes *ApaI* - *SmaI* and dephosphorylated to give the plasmid pYGK-GRE2p.

The plasmid pYGK-GRE2p was then introduced into the strain *E. coli* DH5a to verify the construction. The sequencing of the gene GRE2p of the plasmid pYGK-GRE2p with the oligonucleotides universal M13 reverse and universal M13 forward was then carried out to confirm the construction.

15 The plasmid pRSGK-GRE2p was finally obtained by digestion of the plasmids pYGK-GRE2p and pRS426 with the restriction enzymes *NotI* - *SacII* followed by ligation.

**Example 9: Construction of the strain *S. cerevisiae* [ $\Delta$ (ZTA1) pRSGK-GRE2p] and bioconversion of ethylacetoacetate into ethyl-3-hydroxybutyrate**

20 The inactivation of the gene ZTA1 was carried out by inserting a marker (antibiotic resistance, auxotrophy) while at the same time deleting most of the gene concerned. The technique used is described by Brachmann *et al.* (Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications, *Yeast*, 1998, 14: 115-32). It is also possible to use the method described by Wach *et al.* (New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*, *Yeast*, 1994, 10: 1793-1808).

In all cases a final strain of *S. cerevisiae* [ $\Delta$ (ZTA1)] was obtained into which the plasmid pRSGK-GRE2p (Example 8) was then introduced.

Alternatively, it was also possible to introduce the plasmid pRSGK-GRE2p into an available  $\Delta$ (ZTA1) strain, for example the strain EUROSCARF Y33183 (genotype: BY4743;

Mat  $a/\alpha$ ; *his3D1/his3D1*; *leu2D0/leu2D0*; *lys2D0/LYS2*; *MET15/met15D0*; *ura3D0/ura3D0*; *YBR046c::kanMX4/YBR046c::kanMX4*). It was then possible, after sporulation, to recover a homozygote strain of *S. cerevisiae* [ $\Delta$ (ZTA1) pRSGK-GRE2p].

The strain *S. cerevisiae* [ $\Delta$ (ZTA1) pRSGK-GRE2p] obtained was then grown in a minimum medium containing glucose and ethylacetoacetate.

The control strain *S. cerevisiae* [pRSGK-GRE2p] was grown under the same conditions.

When growth was completed the following variables were compared:

- The time course of the biomass of each strain during the bioconversion phase.
- The quantity of ethyl-3-hydroxybutyrate produced in the extracellular medium.
- The quantity of ethyl-3-hydroxybutyrate accumulated in the cells.
- The productivity in terms of ethyl-3-hydroxybutyrate.
- The yield glucose/ethyl-3-hydroxybutyrate.

We found that the strain *S. cerevisiae* [ $\Delta$ (ZTA1) pRSGK-GRE2p] gave a higher production yield of ethyl-3-hydroxybutyrate than the non-optimized strain.

#### **Example 10: Construction of the strain *S. cerevisiae* [ $\Delta$ (ZTA1, PGI1) pRSGK-GRE2p] and bioconversion of ethylacetoacetate into ethyl-3-hydroxybutyrate**

The inactivation of the gene PGI1 was carried out in the strain *S. cerevisiae* [ $\Delta$ (ZTA1) pRSGK-GRE2p] using the method described in Example 9, and the following oligonucleotides:

Dpgi1F

CCAACGCAGACCGCTGCCTGGCAGGCACTACAGAAACACTTCGATGAAATGAAA  
GACGTTACGATCGCCGATCTTTTGCTGTAGGCTGGAGCTGCTTCG

Dpgi1R

GCGCCACGCTTTATAGCGGTTAATCAGACCATTGGTCGAGCTATCGTGGCTGCTGA  
TTTCTTTATCATCTTTCAGCTCTGCATATGAATATCCTCCTTAG

Pgi1F

GCGGGGCGGTTGTCAACGATGGGGTCATGC

Pgi1R

CGGTATGATTTCCGTTAAATTACAGACAAG

Alternatively it was possible to use an available  $\Delta$ (PGI1) strain, for example the strain EUROSCARF Y23336 (Mat  $\alpha/a$ ; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YBR196c::kanMX4/YBR196c). The strain was then converted by the plasmid pRSGK-GRE2p (Example 8), and the deletion of the gene ZTA1 was then carried out using the method described in Example 9.

The strain *S. cerevisiae* [ $\Delta$ (ZTA1, PGI1) pRSGK-GRE2p] obtained was then grown in minimal medium containing glucose and ethylacetoacetate.

The control strain *S. cerevisiae* [pRSGK-GRE2p] was grown in the same conditions.

When growth was completed the following variables were compared:

- The time course of the biomass of each strain during the bioconversion phase.
- The quantity of ethyl-3-hydroxybutyrate produced in the extracellular medium.
- The quantity of ethyl-3-hydroxybutyrate accumulated in the cells.
- The productivity in terms of ethyl-3-hydroxybutyrate.
- The yield glucose/ethyl-3-hydroxybutyrate.

We observed that the strain *E. coli* [ $\Delta$ (ZTA1, PGI1) pRSGK-GRE2p] gave a higher production yield of ethyl-3-hydroxybutyrate than the non-optimized strain.

#### **Example 11: Construction of the strain *S. cerevisiae* [ $\Delta$ (ZTA1, PFK1, PFK2) pRSGK-GRE2p] and bioconversion of ethylacetoacetate into ethyl-3-hydroxybutyrate**

The genes PFK1 and PFK2 were deleted in the strain *S. cerevisiae* [ $\Delta$ (ZTA1)] using the method described in Example 9, and the following oligonucleotides:

Dpfk1F

ATGCAATCTCAAGATTCATGCTACGGTGTTGCATTCAGATCTATCATCACAAATGATGAAAAGCTTCGTACGCTGCAGGTCTG

Dpfk1R

TTTGTTCAGCGGCTAAAGCGGCTACCTCAGCTCTCAACTTTAATCTACCGGACAGGATGGGCCACTAGTGGATCTGATATC

Pfk1 int F

GCTTTCTTAGAAGCTACCTCCG

Pfk1 int R

GAACCGACAAGACCAACAATGG

5 Pfk2 int F

CAGTTGTACACTTTGGACCC

Pfk2 int R

GATCAGCACCAGTCAAAGAACC

The strain was then converted by the plasmid pRSGK-GRE2p (Example 8).

10 The strain *S. cerevisiae* [ $\Delta$ (ZTA1, PFK1, PFK2) pRSGK-GRE2p] obtained was then grown in minimum medium containing glucose and ethylacetoacetate.

The control strain *S. cerevisiae* [pRSGK-GRE2p] was grown under the same conditions.

When growth was completed the following variables were compared:

- 15
- The time course of the biomass of each strain during the bioconversion phase.
  - The quantity of ethyl-3-hydroxybutyrate produced in the extracellular medium.
  - The quantity of ethyl-3-hydroxybutyrate accumulated in the cells.
  - The productivity in terms of ethyl-3-hydroxybutyrate.
  - The yield glucose/ethyl-3-hydroxybutyrate.

20 We observed that the strain *S. cerevisiae* [ $\Delta$ (ZTA1, PFK1, PFK2) pRSGK-GRE2p] gave a higher production yield of ethyl-3-hydroxybutyrate than the non-optimized strain.

|   | mol <sub>EB</sub> /mol <sub>Glucose</sub> |
|---|---|
| <i>S. cerevisiae</i> [pRSGK-GRE2p]                              | in progress                               |
| <i>S. cerevisiae</i> [ $\Delta$ (ZTA1, PFK1, PFK2) pRSGK-GRE2p] | in progress                               |

**Example 12: Comparison between experimental values and predictions using the metabolic model for the optimization of the production of ethyl-3-hydroxybutyrate by *Escherichia coli***

We found a close correlation between predictive modelling (Example 1) and the experimental results described in Examples 3, 4, 5, 6, 7, 9, 10 and 11.

Examples 1 to 11 above are specific applications of the patent and do not restrict its scope. Those skilled in the art can readily adapt these examples for the biotransformation of substances formed by a NADPH-dependent synthesis. The algorithm MetOpt® and the strategy for optimizing a NADPH-dependent biotransformation process via the optimization of the NADPH/NADP<sup>+</sup> ratio is validated; this patent thus claims an application covering all NADPH-dependent biotransformations that can be modelled and predicted with MetOpt®, or any of its derivatives, using *E. coli*, *S. cerevisiae* or any other microorganism.

**Example 13: Calculation of theoretical optimal yields in fermentation processes in *E. coli***

Example 12 shows that the models MetOpt® developed by the Company are applicable to the bioconversions and should be more generally applicable to biotransformations such as fermentations.

For example, MetOpt®-Coli model was applied to the production of cysteine or 3-hydroxypropionate by the fermentation of glucose in the strains of *E. coli* optimized according to the invention. The parameters used were the same as in Example 1, namely: 1) a glucose import flux of 3 mmol.g<sup>-1</sup>.h<sup>-1</sup>, 2) a variable growth rate of 0, 0.15 and 0.25 h<sup>-1</sup>, 3) a variable membrane-bound transhydrogenase flux (*pntAB*) less than or equal to 1 mmol.g<sup>-1</sup>.h<sup>-1</sup>; the limiting value of membrane-bound transhydrogenase flux was determined from the literature (Hanson, 1979; Anderlund *et al.*, 1999; Emmerling *et al.*, 2002), and 4) maintenance flux limited to between 5 and 22 mmol.g<sup>-1</sup>.h<sup>-1</sup>.

**a) Case of production of cysteine by fermentation of glucose**

|  | $\mu = 0$ | $\mu = 0.15 \text{ h}^{-1}$ | $\mu = 0.25 \text{ h}^{-1}$ |
|--|-----------|-----------------------------|-----------------------------|
| $\Delta(\text{udhA}, \text{qor}, \text{pgi})$  | 0.66      | 0.37                        | 0.09                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pgi})$<br><i>gapA</i> -NADP-dependent   | 0.78      | 0.37                        | 0.09                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pgi})$<br><i>lpd</i> -NADP-dependent  | 0.78      | 0.37                        | 0.09                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pgi})$<br><i>gapA</i> -NADP-dependent<br><i>lpd</i> -NADP-dependent               | 0.78      | 0.37                        | 0.09                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pfkA}, \text{pfkB})$  | 0.40      | 0.18                        | 0.01                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pfkA}, \text{pfkB})$<br><i>gapA</i> -NADP-dependent                               | 0.62      | 0.30                        | 0.06                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pfkA}, \text{pfkB})$<br><i>lpd</i> -NADP-dependent                                | 0.71      | 0.36                        | 0.13                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pfkA}, \text{pfkB})$<br><i>gapA</i> -NADP-dependent<br><i>lpd</i> -NADP-dependent | 0.77      | 0.42                        | 0.17                        |

**Theoretical optimal yields for the production of cysteine by strains of *E. coli* optimized for NADP<sup>+</sup> reduction capacity (mol per mol of glucose)**

5 To further improve the theoretical optimal yield of the strains optimized according to the invention additional modifications can be made, such as the overexpression of at least one gene that can be *zwf*, *gnd*, *pntA*, *pntB* or *icd* and/or the deletion of at least one gene that can be *edd*, *aceA*, *aceB* or *aceK*.

10 In practice, to obtain such yields other modifications have to be made to the strains optimized according to the invention, for example by overexpressing the gene *cysB* as described in the patent WO0127307, or by modifying the gene *cysE* as described in the patent EP0885962.

**b) Case of production of 3-hydroxypropionate by fermentation of glucose**

15 The production of 3-hydroxypropionate was carried out in strains of *E. coli* containing the genes coding for the enzymes of the 3-hydroxypropionate synthesis pathway, for example the malonyl-coA reductase of *Chloroflexus aurantiacus* (Hügler et al., *Journal of Bacteriology*, 2002, 184: 2404-2410).

|  | $\mu = 0$ | $\mu = 0.15 \text{ h}^{-1}$ | $\mu = 0.25 \text{ h}^{-1}$ |
|--|-----------|-----------------------------|-----------------------------|
| $\Delta(\text{udhA}, \text{qor}, \text{pgi})$  | 1.33      | 0.79                        | 0.30                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pgi})$<br><i>gapA</i> -NADP-dependent   | 1.76      | 0.99                        | 0.30                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pgi})$<br><i>lpd</i> -NADP-dependent  | 1.82      | 0.99                        | 0.30                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pgi})$<br><i>gapA</i> -NADP-dependent<br><i>lpd</i> -NADP-dependent               | 1.82      | 0.99                        | 0.30                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pfkA}, \text{pfkB})$  | 1.62      | 0.66                        | 0.03                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pfkA}, \text{pfkB})$<br><i>gapA</i> -NADP-dependent                               | 1.76      | 0.79                        | 0.07                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pfkA}, \text{pfkB})$<br><i>lpd</i> -NADP-dependent                                | 1.79      | 0.84                        | 0.07                        |
| $\Delta(\text{udhA}, \text{qor}, \text{pfkA}, \text{pfkB})$<br><i>gapA</i> -NADP-dependent<br><i>lpd</i> -NADP-dependent | 1.79      | 0.84                        | 0.07                        |

**Theoretical optimal yields for the production of 3-hydroxypropionate by strains of *E. coli* optimized for NADP<sup>+</sup> reduction capacity  
(mol per mol of glucose)**

5

To further improve the theoretical optimal yield of the strains optimized according to the invention, additional modifications can be made, such as the overexpression of at least one gene that can be *zwf*, *gnd*, *pntA*, *pntB* or *icd* and/or the deletion of at least one gene that can be *edd*, *aceA*, *aceB* or *aceK*.

10

**Example 14: Calculation of theoretical optimal yields in fermentation processes in *S. cerevisiae*; application to the production of hydrocortisone**

Example 12 shows that the MetOpt® models developed by the Company are applicable to bioconversions and should be more generally applicable to biotransformations such as fermentations.

For example, the MetOpt®-Scere model was applied to the production of hydrocortisone by fermentation of glucose in the strains of *S. cerevisiae* optimized according to the invention. The parameters used were the same as in Example 1, namely: 1) a glucose



import flux of 3 mmol.g<sup>-1</sup>.h<sup>-1</sup>, 2) a variable growth rate of 0, 0.15 and 0.25 h<sup>-1</sup>, 3) a maintenance flux less than or equal to 22 mmol.g<sup>-1</sup>.h<sup>-1</sup>, 4) reactions of aldehyde dehydrogenases (ALD2, ALD3, ALD6) irreversible and set in the direction acetate + NAD(P)H → acetaldehyde + NAD(P), and 5) no activities equivalent to *udhA* or *pntA,B*.

5 The model allows for mitochondrial and peroxisomal compartmentalization.

This representation of the results demonstrates the real contribution made by each of the mutations made according to the invention to the improvement in NADPH production and so to the improvement in the hydrocortisone production flux.

10 The production of hydrocortisone was achieved in strains of *S. cerevisiae* containing genes coding for enzymes of the hydrocortisone synthesis pathway (Szczębara et al., 2003, *Nature Biotechnology*, 21: 143-149).

|  | $\mu = 0$ | $\mu = 0.15 \text{ h}^{-1}$ | $\mu = 0.25 \text{ h}^{-1}$ |
|--|-----------|-----------------------------|-----------------------------|
| $\Delta(\text{ZTA1,PGI1})$   | 0.12      | 0.08                        | 0.06                        |
| $\Delta(\text{ZTA1,PGI1})$<br>TDH1,2,3-NADP-dependent                        | 0.21      | 0.14                        | 0.10                        |
| $\Delta(\text{ZTA1,PGI1})$<br>LPD1-NADP-dependent                            | 0.20      | 0.14                        | 0.10                        |
| $\Delta(\text{ZTA1,PGI1})$<br>TDH1,2,3-NADP-dependent<br>LPD1-NADP-dependent | 0.21      | 0.14                        | 0.10                        |

**Theoretical optimal yields for the production of hydrocortisone by strains of *E. coli*  
15 optimized for NADP<sup>+</sup> reduction capacity  
(mol per mol of glucose)**

20 The strains from which the genes PFK1 and PFK2 have been deleted are unable to produce hydrocortisone, and may not even be viable. This is because the production of hydrocortisone is limited more by carbon demand than by NADPH requirement. One solution is to allow a weak expression of a transhydrogenase type activity in the yeast. However, modelling shows that the hydrocortisone production will never be as high as when the PGI1 gene is deleted.

To further improve the theoretical optimum yield of the strains optimized according to the invention, additional modifications can be made, such as the overexpression of at least one

gene that can be ZWF, SOL1, SOL2, SOL3, SOL4, GND1, GND2, IDP1, IDP2 or IDP3, and/or the deletion of at least one gene that can be either ICL1 or DAL7.

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